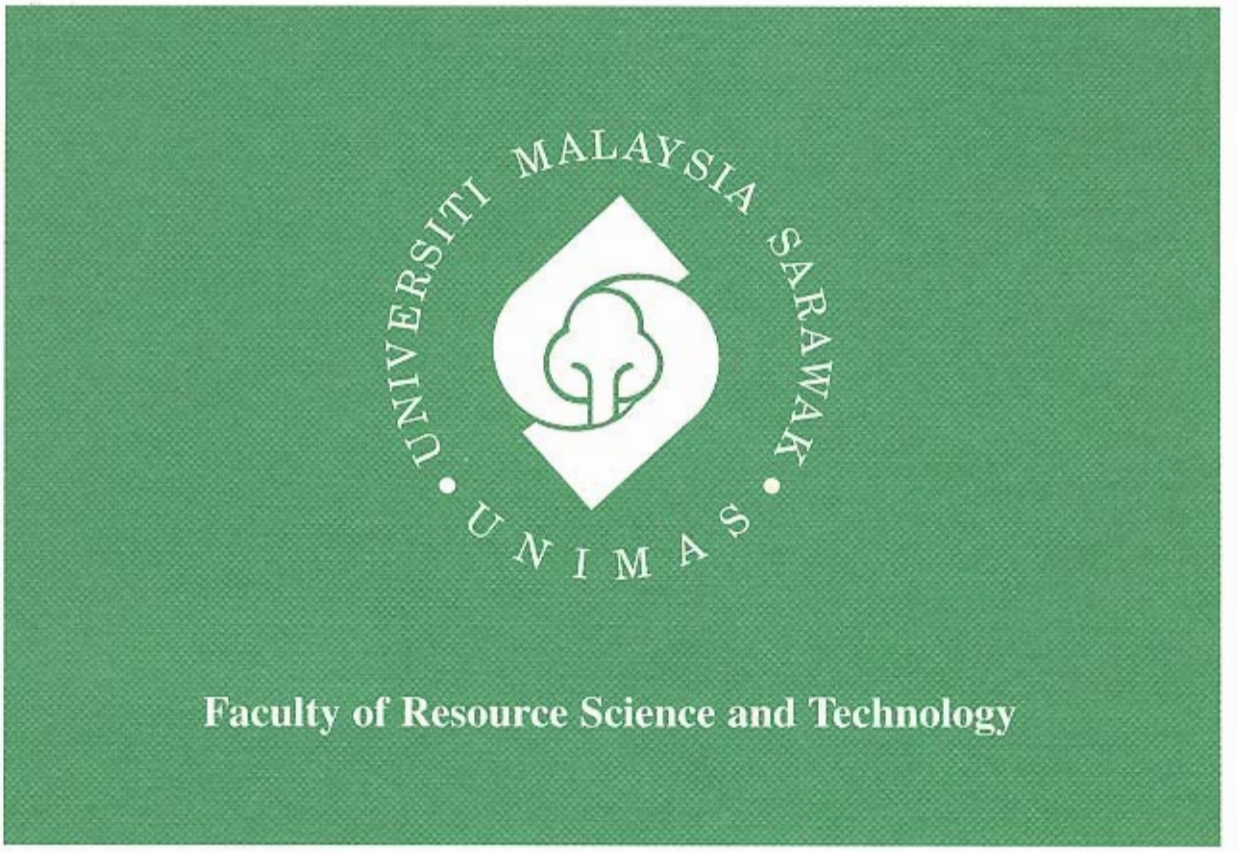

GENETIC VARIATION WITHIN THE GENUS *Cynopterus* (CHIROPTERA: PTEROPODIDAE) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE

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Bachelor of Science with Honours
(Animal Resource Science and Management)
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DECLARATION

I hereby declare that no portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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ABSTRACT

Random Amplified Polymorphic DNA (RAPD) molecular marker was used as a tool in screening the genetic variation within bats of the genus *Cynopterus*. Bat species that were used in the study were *C. brachyotis* (Sunda and Forest) and *C. horsfieldi*. A total of 12 primers were tested but only OPD–04 was selected as diagnostic marker since it demonstrates to be most polymorphic and presumably suitable as genetic marker for *Cynopterus* species. The diagnostic marker was able to distinguished *C. brachyotis* and *C. horsfieldi* including the cryptic species within the *C. brachyotis* complex. Bands having the size of 800 bp and 900 bp were present and shared in *C. brachyotis* Forest and *C. horsfieldi*, indicating that the two are closely related to each other compare to *C. brachyotis* Sunda and *C. horsfieldi*.

Key words: Random Amplified Polymorphic DNA, bats, *Cynopterus*, genetic variation, diagnostic marker

ABSTRAK

Teknik analisis secara rawak DNA polimorfik (RAPD) telah digunakan sebagai alat untuk memeriksa variasi genetik kelawar daripada genus Cynopterus. Spesies kelawar yang digunakan dalam kajian ini ialah C. brachyotis (Sunda dan Forest) dan C. horsfieldi. Sebanyak 12 primer telah diuji tetapi hanya OPD–04 dipilih sebagai penanda diagnostik kerana ia terbukti sebagai paling polimorfik dan dianggap sesuai dijadikan sebagai primer diagnostik untuk spesies Cynopterus. Primer diagnostik ini mampu membezakan C. brachyotis dan C. horsfieldi termasuk spesies kriptik dalam kompleks C. brachyotis. Jalur bersaiz 800 bp dan 900 bp hadir dan dikongsi bersama C. brachyotis Forest dan C. horsfieldi dan menunjukkan bahawa kedua-duanya mempunyai hubungan rapat berbanding C. brachyotis Sunda dan C. horsfieldi.

Kata kunci: *Teknik analisis secara rawak DNA polimorfik (RAPD), kelawar, Cynopterus, variasi genetic, penanda diagnostik*

1.0 INTRODUCTION

1.1 Study Background

Bats are placed in the Order Chiroptera (Corbet and Hill, 1992) and are divided into two suborders, the Old World Megachiroptera and Microchiroptera (Findley, 1993). They are the only mammals that possess true flight (Whittow, 1983) and are widely distributed in the tropical or subtropical region where variety of foods and habitats are found there (Corbet and Hill, 1992). Their fingers are modified and elongated, with skin membranes between these fingers to create a wing surface (Payne *et al.,* 1985). In Malaysia, they are the largest group of mammals in terms of number of species and total number of individuals compare to other mammal groups occur here (Whittow, 1983).

The megachiropterans are bats having a predominantly frugivorous or nectarivorous diets while the microchiropterans are the insect eating bats although some do catch and eat fishes, fiddler crabs and even do blood-sucking (Altringham, 1996). The megachiropterans are considered as to be more primitive than the microchiropterans and they lack laryngeal echolocation except for bats in the genus *Rousettus* (Whittow, 1983; Payne *et al.*, 1985). The megachiropterans consist of a single family, the Pteropodidae, with about 200 species (Koopman, 1994). Generally, the pteropodids are bats with large conspicuous eyes, dog-like muzzle, having well-developed second finger and presence of a claw on the first and second finger except in *Eonycteris* (Tweedie, 1978; Payne *et al.* 1985).

The megachiropterans can be divided into two main groups; the macroglossine bats and the cynopterine bats. According to Anderson (1912), the Cynopterine tribe of the family Pteropodidae comprises of 11 genera, but only five genera can be found in Malaysia namely, *Balionycteris, Dyacopterus, Chironax, Penthetor* and *Cynopterus*. The genus *Cynopterus* have a dog-liked face appearance and they are widely distributed in the Indo-Malayan region (Corbet and Hill, 1992). They are medium to large bats, having short and stout muzzle and their furs are yellowish or reddish in colour with whitish wing bones and rim to the ears (Payne *et. al.,* 1985).

In the Indo-Malayan region, there are generally five species of *Cynopterus* recognized and occurred here namely *C. brachyotis*, *C. horsfieldi*, *C. sphinx*, *C. titthaecheilus* and *C. nusatenggara* (Wilson and Reeder, 1993). Out of the five species, only three species occurs in Malaysia, namely, *C. brachyotis, C. horsfieldi* and *C. sphinx* (Lim, 1966; Medway, 1978; Payne *et. al.,* 1985; Corbet and Hill, 1992). These three species are widely spread and shows sympatric distribution in the Southeast Asia mainland, the Malay Peninsula, Borneo and Sumatra and also in India and Sri Lanka (*C. brachyotis* and *C. sphinx*) as shown in Appendix 1.

The short-nosed fruit bat (*C. sphinx*) and the greater short-nosed fruit bat (*C. brachyotis*) are the most geographically dispersed members of the genus *Cynopterus* (Corbet and Hill, 1992; Storz and Kunz, 1999; Storz and Beaumont, 2002; Campbell *et al.* 2004) with the latter being the commonest and widely spread among the fruit bats in the tropical Asian region (Lim, 1966; Lekagul and McNeely, 1977; Medway, 1978; Boon and Corlett, 1989). *C. sphinx* is

widely distributed in the tropical South Asia and peripherally along the Malay Archipelago western margin (Storz and Kunz, 1999; Storz and Beaumont, 2002) while the Horsfield's fruit Bat (*C. horsfieldi*) is restricted to the Malay Peninsula and in the islands of Sumatra, Java and Borneo (Corbet and Hill, 1992; Koopman, 1994; Campbell *et al.* 2004; Campbell *et al.* 2006).

For the past few years, advancement in molecular biology has given us the access to the entire genome, but at rather heavily techniques in molecular sorting and polymorphisms visualization (Feral, 2002). Molecular genetic techniques have proven to be very valuable tools in the studies of population, behavioural and evolutionary biology where other methods such as direct observation of individuals or a population is greatly limited (Burland and Wilmer, 2001; Rajan and Marimuthu, 2006). Another advantage in using molecular genetic techniques is the ability of molecular data in comparing organisms that are morphologically very different or very similar (Simmons, 2000).

The development of Polymerase Chain Reaction (PCR) has greatly contributed to the research of eukaryotic genome including development and application of various markers (Marle-Koster and Nel, 2003; Salem *et al.*, 2005). PCR has become a basic and necessary tool for both research and analytical laboratories in detection of pathogens; identify mutations that caused inherited diseases and DNA fingerprinting for medical and forensic reasons (Newton and Graham, 1997). The increasing development of PCR methods has rapidly increased the popularity usage of DNA technologies in detecting genetic variation in natural populations although these methods are rather labour-intensive and costly (Silva and Russo,

2000). Genetic variation generated by molecular markers may provide important information on population structure, levels of gene flow, phylogenetic relationships, patterns of historical biogeography and analysis of parentage and relatedness (Feral, 2002).

Genetic markers can be divided into two: codominant and dominant markers. Codominant markers are markers that enable us to distinguish homozygotes and heterozygotes (Parker *et al.* 1998; Silva and Russo, 2000). Examples of codominant markers are microsatellites, nuclear PCR-RFLP (Restriction Fragment Length Polymorphism) and also allozymes. Dominant markers include Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP). Codominant markers allow calculations of the allelic frequencies directly from the raw data. On the contrary, dominant markers require one to assume that the studied population is in Hardy-Weinberg equilibrium (Silva and Russo, 2000) and does not allow estimation of allele frequencies to be done.

RAPD is among the many molecular tools which provide most information and widely used especially in revealing genetic variation within species in the vertebrates (Moreira and Morielle-Versute, 2006). It is a technique which involves the usage of short (8–10 base pairs), arbitrary oligonucleotide primers in the Polymerase Chain Reaction (PCR) and separation of polymorphic DNA generated through gel electrophoresis (Williams *et al.*, 1990; Welsh and McClelland, 1990). Primers are used singly in one PCR reaction, not in combination with a second primer like the usual standard PCR. RAPD process reveals several polymorphic genetic segments per primer and the degree of polymorphism depend on the nucleotide sequence, secondary structure and number of primers used.

RAPD can be used in wide variety of applications since it is more cost-effective, no required knowledge of species' genome, less labour intensive and requires only a small amount of undegraded genomic DNA compare to other molecular techniques. RAPD markers can be applied for genetic mapping, for plant and animal breeding applications and also for DNA fingerprinting in population genetics studies (Williams *et al.,* 1990; Carlson *et al.,* 1991; Scott *et al.,* 1992; Hadrys *et al.,* 1993; Sinclair *et al.,* 1996; Schilliro *et al.,* 2001; Die, 2005). Also, RAPD enables testing of large number of markers and allows efficient assay for polymorphisms which leads to rapid identification and isolation of chromosome-specific DNA fragments (Williams *et al.,* 1990). Unlike RFLP, RAPD is a much more rapid procedure where it does not require lengthy extraction and avoids usage of radioactive labels in Southern blot hybridizations (Ismail, 1998; Bardacki, 2001).

1.2 Statement of Problem

The ambiguous taxonomic status within the genus *Cynopterus* has been a subject of controversy for many decades especially with regard to the *C. brachyotis* species complex (Anderson, 1912; Payne *et. al.,* 1985; Corbet and Hill, 1992; Storz and Kunz, 1999; Storz and Beaumont, 2002; Mapatuna *et al.,* 2002; Campbell *et al.,* 2004; Campbell *et al.,* 2006). Although there are a quite number of studies that had been conducted to resolved the taxonomic status of the *Cynopterus* species, the matter has been much debated by several authors and has been subject to a number of revisions based on morphological characters such as skeletal, cranial and external characters.

The problem in assigning and differentiating the two as separate species arises due to the particularly high morphological variations that occur within the *C. brachyotis* populations which differ among the geographic localities and habitat types (Payne *et. al.,* 1985; Corbet and Hill, 1992; Abdullah *et al.,* 2000; Mapatuna *et al.,* 2002; Abdullah, 2003; Campbell *et al.,* 2004). Therefore this study was conducted to characterize the genetic variability for *C. brachyotis* and *C. horsfieldi* and to identify a suitable species-specific marker for bats within the genus *Cynopterus*.

Thus, the present study was conducted to screen for genetic variation and determined if the selected primer can be used as a diagnostic marker for *Cynopterus* species using RAPD.

2.0 LITERATURE REVIEW

2.1 Study Taxon

Morphological variations occur within the *C. brachyotis* has been earlier noted by Payne *et al.* (1985). According to Payne *et al.* (1985), the tall forest dwelling *C. brachyotis* are found to be significantly smaller in size compare to the typical forest edge and gardens *C. brachyotis*, although there are some overlapping between the two forms. Francis (1990) also recorded that the body size of forest dwelling *C. brachyotis* is smaller than the forest edge *C. brachyotis*. Recent studies conducted by Julaihi (2005), Jayaraj *et al.* (2004), Campbell *et al.* (2004), Abdullah (2003) and Abdullah *et al.* (2000) supported the occurrence of at least two cryptic species within the *C. brachyotis* populations based on morphological and genetic differences observed.

In a study conducted by Abdullah (2003), the occurrence of two cryptic species within the *C. brachyotis* populations is strongly supported based on the mitochondrial DNA cytochrome *b* gene phylogenetic tree. The generated tree reveals a major split within the *C. brachyotis* and is supported with 100% bootstrap value. However, it is found that the large form *C. brachyotis* is monophyletic, which contradicts with the present taxonomy. The small form *C. brachyotis* is found to be closely related to *C. horsfieldi* and *C. sphinx*.

Campbell *et al.* (2004) had used mitochondrial DNA sequence data from the control region and cytochrome *b* to investigate the phylogenetic relationship of *C. brachyotis*, *C. horsfieldi*

and *C. sphinx*. Based on the study, Campbell *et al.* (2004) classified the two cryptic species within the *C. brachyotis* populations as *C. brachyotis* Sunda and *C. brachyotis* Forest based on their ecology, morphology and genetic divergent lineages. *C. brachyotis* Sunda represents the larger form *C. brachyotis* while *C. brachyotis* Forest refers to the smaller form *C. brachyotis*. The designation of the two cryptic species was based on the phylogenetic analyses which show *C. brachyotis* is a complex of distinct evolutionary lineages, which includes *C. horsfieldi* and *C. sphinx*.

In contrast of the controversies and confusion regarding the taxonomic status of *C. brachyotis* and *C. sphinx*, the taxonomic status of *C. horsfieldi* has not been questioned since the species can be distinguished from other species of *Cynopterus* based on the its large body size and dental morphology (Payne *et al.*, 1985; Corbet and Hill, 1992; Koopman, 1994). According to Payne *et al.*, (1985), *C. horsfieldi* is characterized by having broader and squarer cheek teeth and presence of cusps at the last lower premolar and first lower molar which is absence in the other two species.

2.2 Random Amplified Polymorphic DNA (RAPD)

In a study conducted by Almeida *et al.* (2000), RAPD analysis was used to reveal the genetic distance and population genetic structure of water rats, *Nectomys squamipes* in Brazil. In this study, 45 scorable bands were produced by three primers, of which 31 were polymorphic bands. The study revealed that there was a significant differentiation among the populations studied with the estimated FST value was 0.17. This study also showed that the RAPD

procedures as useful tool in determining genetic variability since it generates reproducible band profiles and the inheritance pattern proved to be Mendelian for a dominant marker.

Barnabe *et al.* (2003) has used RAPD technique for phylogenetic analysis of trypanosome bats of the subgenus *Schizotrypanum*. These bats are very hard to differentiate morphologically. Based on the study, RAPD has clearly distinguished these cryptic species and the phylogenetic analysis based on RAPD data agrees with the species attribution proposed by Baker *et al.* (1978). Therefore, RAPD can be used for taxonomic assignment and provide valuable phylogenetic information of the studied species.

Abu-Hassan (2005) has also used RAPD technique in identifying genetic markers for *Barbonymus schwanenfildii* (Cyprinidae) and *Barbonymus gonionotus* (Cyprinidae). The study revealed high percentage of allelic frequencies for all the polymorphic loci from the *B. schwanenfildii* and *B. gonionotus* populations, which is within the ranged of 55.56% to 88.89%. This study also revealed that PCR-RAPD were useful in illustrating the genetic relatedness of the two species and in identification of genetic markers.

Recent study by Moreira and Morielle-Versute (2006) utilized RAPD to determine genetic variability in seven species of bats belonging to five genera. These bats are *Molossus molossus*, *M. rufus*, *Eumops glaucinus*, *E. perotis*, *Myotis nigricans*, *Eptesicus furinalis* and *Artibeus planirostris*. A total of 20 decanucleotide primers were used and the genetic diversity was determined based on 741 bands produced by the primers. The Nei gene diversity index within the species obtained was lower than 0.22, indicating a geneti

conservatism in the seven species. The study also shows that RAPD-PCR assay is capable of revealing polymorphism in species of bats.

3.0 MATERIALS AND METHODS

3.1 Sample collection and species identification.

A total of 12 individuals of bats (genus *Cynopterus*) were used in this study. Preserved or fresh samples were obtained from various localities in Peninsular Malaysia and Sarawak (Table 1). Tissue samples were obtained from the chest muscle of each sample and preserved in 95% ethanol and kept in a freezer at –80°C for DNA analysis. Two species of the genus *Cynopterus* that were used in this study are *C. brachyotis* (two forms; Sunda and Forest) and *C. horsfieldi*.

Identification of species was done according to Payne *et al.* (1985). Forearm measurement for *C. brachyotis* Forest is within the range of 55 to 63 mm and *C. brachyotis* Sunda 64 to 67 mm (Campbell *et al.* 2004 whereas for *C. horsfieldi* is within 68 to 76 mm. Identification of species were also based on the shape of their cheek teeth and presence of accessory cusps. Immature and adult bats were distinguished from each other by looking at the wing joints of the bats against a bright light or torchlight. Immature wing joints appear banded due to the cartilage has not fully turned to bone while adult joints appear as solid lump. However, *C. sphinx* was not used in this study due to inaccessibility to obtain fresh sample, limited museum samples available and time constraint factor.

Table 1. Species name, forearm measurement, sex and locality used in the study.

No.	Species	Coding	Forearm (mm)	Sex	Locality
1.	*C. brachyotis*	PS071	63.40	M	Pulau Satang
2.	*C. brachyotis*	PS073	53.49	F	Pulau Satang
3.	*C. brachyotis*	PS075	57.83	M	Pulau Satang
4.	*C. brachyotis*	PS145	66.36	F	Pulau Satang
5.	*C. brachyotis*	BNP241	65.04	M	Bako National Park
6.	*C. brachyotis*	M1	64.16	F	Jambusan Cave
7.	*C. brachyotis*	RV37	59.10	M	Gunung Murut
8.	*C. brachyotis*	RV40	56.54	F	Gunung Murut
9.	*C. horsfieldi*	V3	77.60	M	Kubah National Park
10.	*C. horsfieldi*	SD24	79.47	M	Sungai Dusun, Selangor
11.	*C. horsfieldi*	TL6	70.0	M	Taleban, Thailand
12.	*C. horsfieldi*	JC70	79.10	F	Jambusan Cave

3.2 DNA extraction

Total genomic DNA was extracted from both fresh and preserved tissue muscles according to modified cetyltrimethylammonium bromide (CTAB) protocol based on Grewe *et al.* (1993). Finely minced tissue samples were transferred into a 1.5 mL microcentrifuge tube containing 700 µL of CTAB. Next, 5µL of Proteinase K were added into the microcentrifuge tube and incubated in waterbath at 60°C until a clear mixture obtained.

The microcentrifuge tube was then added with 700 µL of chloroform isoamyl-alcohol, vortexed and centrifuged for 15 minutes at 13,000 rpm. Two layers of liquid were seen in the tube and upper aqueous layer (supernatant) were transferred into a new 1.5 mL microcentrifuge tube. Next, 600 µL of absolute ethanol was added into the tube, vortexed and centrifuged at 13,000 rpm for 15 minutes. The ethanol supernatant was carefully discarded, leaving only the pellet in the tube.

Then, 600 µL of 70% ethanol and 25 µL of 3M sodium chloride (NaCl) were added into the tube, vortexed and centrifuged for 15 minutes at 13,000 rpm. The supernatant were carefully discarded again to ensure the pellet was dried and excess ethanol was removed. Lastly, 25–30 µL of de-ionized water were added into the tube-containing pellet and stored at –20 °C until further use.

3.3 Polymerase Chain Reaction-(PCR) Randomly Amplified Polymorphic DNA (RAPD)

3.3.1 PCR-RAPD

RAPD reactions were carried out based on a modified protocol from Williams *et al.* (1990). PCR-RAPD amplifications were performed in a 24 µL reaction mixture and the contents of the reaction mixture are shown in Table 2. Negative control with no DNA template for each PCR reaction was used to check for contamination.

Table 2. PCR reaction mixture.

Reagents	24 µL
10X buffer	2.5
$MgCl_2$ (25mM)	1.5
dNTP (2mM)	0.5
Primer (10µM)	1.0
ddH_2O	17.3
DNA template	1.0
Taq polymerase (5 units/µL)	0.2

The thermal cycling profile used in the present study was based on a study done by Moreira and Morielle-Versute (2006) with some modifications. PCR thermal cycling parameters are shown in Table 3. PCR amplifications were done using the Biometra T-personal thermalcycler.

Table 3. PCR thermal cycling parameters.

Step	Temperature (°C)	Time	No. Cycles
Pre-denaturation	94	2 min	
Denaturation	94	15 s	43
Annealing	35	30 s	
Extension	72	2 min	
Final extension	72	5 min	

3.3.2 Primer

A set of 13 arbitrary primers (Kit D) from Operon Technologies Inc., Alameda, California, USA with 60–70% GC content were used to screen for any polymorphisms occur within the *Cynopterus* species in this study (Table 3).

Table 4. Primers and primer sequences used in this study.

Primer	Sequence	GC- content (%)
OPD-01	5'-ACCGCGAAGG	70
OPD-02	5'-GGACCCAACC	70
OPD-04	5'-TCTGGTGAGG	60
OPD-06	5'-ACCTGAACGG	60
OPD-07	5'-TTGGCACGGG	70
OPD-09	5'-CTCTGGAGAC	60
OPD-10	5'-GGTCTACACC	60
OPD-11	5'-AGCGCCATTG	60
OPD-12	5'-CACCGTATCC	60
OPD-13	5'-GGGGTGACGA	70
OPD-14	5'-CTTCCCCAAG	60
OPD-15	5'-CATCCGTGCT	60
OPD-16	5'-AGGGCGTAAG	60

3.4 Agarose Gel Electrophoresis

DNA extraction products were electrophoresed on a 1% agarose gel (1st Base Biotechnology Grade) stained with ethidium bromide in 1X TAE buffer. One micro litre of 6X loading dye solution (Fermentas) was loaded with 2μL of DNA sample. A 2μL of Generuler™ 1kb DNA Ladder (Fermentas) was used as a standard size marker.

RAPD-PCR products were electrophoresed on a 2.0% agarose (1st Base Biotechnology Grade) gel stained ethidium bromide in 1X TAE buffer. For RAPD-PCR product visualization, one micro litre of 6X loading dye solution (Fermentas) was loaded with 5μL of RAPD-PCR product. Two micro litre of Generuler™ 100bp DNA Ladder (Fermentas) was used to estimate the fragment size.

Gels were run for at least two hours at 90V. Gels were visualized and photographed using the Gel Doc 1000 Gel Documentation System (Bio-Rad).